Effect of hyperglycaemic conditions on the response of human periodontal ligament fibroblasts to mechanical stretching

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Abstract: Objectives: The aim of the present study was to investigate the impact of high glucose concentration on the response of human periodontal ligament fibroblasts (PDLFs) to cyclic tensile strain. Materials and methods: Human PDLFs were incubated under normal or high glucose conditions, and then were subjected to cyclic tensile stretching (8 per cent extension, 1 Hz). Gene expression was determined by quantitative real-time polymerase chain reaction. Intracellular reactive oxygen species (ROS) were determined by the 2',7'-dichlorofluorescein-diacetate assay, activation of mitogen-activated protein kinase (MAPK) was monitored by western analysis and osteoblastic differentiation was estimated with Alizarin Red-S staining. Results: Cyclic tensile stretching of PDLF leads to an immediate activation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), as well as to the increased expression of the transcription factor c-fos, known to regulate many osteogenesis-related genes. At later time points, the alkaline phosphatase and osteopontin genes were also upregulated. Hyperglycaemic conditions inhibited these effects. High glucose conditions were unable to increase ROS levels, but they increased the medium's osmolality. Finally, increase of osmolality mimics the inhibitory effect of hyperglycaemia on MAPK activation, c-fos and osteoblast-specific gene markers' upregulation, as well as osteogenic differentiation capacity. Conclusion: Our findings indicate that under high glucose conditions, human PDLFs fail to adequately respond to mechanical deformation, while their strain-elicited osteoblast differentiation ability is deteriorated. The aforementioned effects are most probably mediated by the increased osmolality under hyperglycaemic conditions.

DOI: https://doi.org/10.1093/ejo/cjz051

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Eur J Orthod. 2019 Nov 15;41(6):583-590. doi: 10.1093/ejo/cjz051.

Abstract

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Materials and Methods: Human PDLF were incubated under normal or high glucose conditions, and then were subjected to cyclic tensile stretching (8% extension, 1 Hz). Gene expression was determined by quantitative real-time polymerase chain reaction (qRT-PCR). Intracellular reactive oxygen species (ROS) were determined by the DCFH-DA assay, activation of MAPK was monitored by Western analysis and osteoblastic differentiation was estimated with Alizarin Red-S staining.

Results: Cyclic tensile stretching of PDLF leads to an immediate activation of ERK and JNK, as well as to the increased expression of the transcription factor *c-fos*, known to regulate many osteogenesis-related genes. At later time-points the alkaline phosphatase and osteopontin genes were also up-regulated. Hyperglycemic conditions inhibited these effects. High glucose conditions were unable to increase ROS levels, but they increased the medium's osmolality. Finally, increase of osmolality mimics the inhibitory effect of hyperglycemia on MAPK activation, *c-fos* and osteoblast-specific gene markers' up-regulation as well as osteogenic differentiation capacity.

Conclusion: Our findings indicate that under high glucose conditions human PDLF fail to adequately respond to mechanical deformation while their strain-elicited osteoblast differentiation ability is deteriorated. The aforementioned effects are most probably mediated by the increased osmolality under hyperglycemic conditions.

Keywords: Hyperglycemia; periodontal ligament fibroblasts; mechanical stretching; osteoblastic differentiation.

Introduction

Diabetes mellitus is a chronic metabolic disease that is characterized by high glucose blood levels and is accompanied by long-term and life-threatening effects, while it is also involved in impaired bone metabolism, in declined quantity and deteriorated quality of the bones (1). Consequently, diabetes is associated with high fracture risk, as well as with a delayed fracture healing (2). Furthermore, the association of the disease with the decrease in the expression of transcription factors that regulate osteoblast differentiation has also been revealed (3). In this context, it has been suggested that diabetes exerts its negative effects through a variety of mechanisms including increased reactive oxygen species (ROS) (4-6), or an osmotic response (7). In fact, the syndrome of hyperosmolar hyperglycemic state has been characterised by a plasma glucose level greater than 600mg/dL, and by a plasma osmolarity exceeding 320mOsm/L (8). However, the mechanism accounting for diabetes' associated bone loss or deterioration is not completely understood.

Periodontal ligament (PDL) is a highly specialized, collagen rich connective tissue positioned between the tooth-root cementum and the alveolar bone. Due to its location comprises the primary target of mechanical forces applied during occlusion and mastication, as well as during therapeutic orthodontic tooth movement (9-11). PDL fibroblasts (PDLF), the prevailing cell residents of this tissue, are the recipients and sensors of such stimuli and respond to them through activated metabolism and proliferation (12-13) or an osteoblastic differentiation (14-15), thus contributing to the maintenance of tissue homeostasis, repair and remodelling.

More specifically, mechanical loading exerted to the teeth and hence to the periodontal ligament is converted into a cellular response by a complex network of mechanical sensing molecules that leads to the activation of signalling pathways (i.e. mechanotransduction), including the family of mitogen-activated protein kinases (MAPK) cascades (i.e. the ERK, p38 and JNK). These pathways activate several transcription factors, such as members of the *fos* family, which regulate the expression of osteoblast-specific genes such as alkaline phosphatase (*ALP*), osteopontin (*OPN*), osteocalcin (*OCN*) and collagen type 1 (*Col1*) (16-17).

Orthodontic tooth movement in the presence of hyperglycemic pattern and potentially associated manifestations has not been explored in the broader orthodontic

literature. The relevant biomedical literature is abundant with evidence indicating that diabetes is manifested through connective tissue disorder which results in the mechanical properties of ligaments in general being deteriorated or with inferior mechanical properties. Research on this topic has shown that the toughness and ultimate stress of the Achilles tendon were lower in diabetic rats suggesting an inhibition of collagen synthesis in tendon fibroblasts (18). Hence the suppressed collagen synthesis may cause the deterioration of the tendon mechanical properties. These phenomena might be one of the reasons of the tendon deterioration in the diabetic patients. Overall, diabetes alters mechanical properties and the dynamic response to load. As diabetes is among the primary risk factors for periodontal disease (2), and given that mechanical forces exerted to periodontal ligament, such as those applied to the tissue during orthodontic treatment, affect osteoblastic differentiation (14-15), aim of the present work was to investigate the short-term effects of high glucose concentration, simulating diabetes mellitus' biological milieu, on the response of human PDL fibroblasts to mechanical deformation, in terms of activation of signalling pathways and transcription factors related to stimulation of osteoblastic differentiation and expression of osteogenic markers, as well as of the underlying mechanism of this effect. The clinical implication of the involvement of PDL tissue in altering its response to loading under the hyperglycemic conditions is obvious for a big number of adolescents and adults seeking orthodontic treatment.

Materials and Methods

Cells and culture conditions

Human teeth of consenting healthy normal donors, with age ranging from 9 to 20 years old, were extracted in the course of orthodontic treatment, after obtaining the approval of the Institute's Bioethics Committee (No 240/2013-1640). PDL tissue explants were used to develop primary cultures of fibroblasts (PDLF), as previously described (19). The cells released from these tissues were cultured in Dulbecco's modified Eagle's medium (DMEM; normal glucose formulation, i.e. 5.5mM), supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) (both from Biochrom AG, Berlin, Germany) and 10% (v/v) fetal bovine serum (FBS, Gibco

BRL, Invitrogen, Paisley, UK) in an environment of 5% CO_2, 85% humidity at 37°C. In order to simulate the hyperglycemic conditions of the diabetic state *in* vitro PDLF were grown in DMEM with high glucose formulation (30mM, HG) supplemented with 10% (v/v) FBS. Adaptation of the concentration of normal glucose medium to 30mM was achieved by adding the appropriate volume of a 2M stock solution of D-glucose into cells' medium. Cells were subcultured using a trypsin/citrate solution (0.25%/0.30%, w/v) solution. Early-passage PDLF of five different donors were used in this study and each experiment has been reproduced in cells from at least two donors.

Hyperosmotic treatment

The osmolality of normal glucose medium (5.5 mM) used was 300 mOsm/kg, while the osmolality of high glucose medium (30 mM) was 330 mOsm/kg, as estimated by freezing-point depression with an Osmomat 030 automatic cryoscopic osmometer (Gonotec, Berlin, Germany). In order to assess whether the increased osmolality caused by high glucose concentration could impact cyclic tensile-stretched PDL fibroblasts' immediate responses cells were exposed to another hyperosmotic agent of different nature, i.e. a salt solution of 5M NaCl and 0.4M KCl, at the same final osmolality (330 mOsm/kg) , as previously reported (20).

Application of cycle tensile stress

Cycle tensile loading was applied to human PDLF with a specially designed device equipped with a rotary motor and a camshaft. Strain magnitude could be modified by the eccentricity of the cams on the rotating motor axis and frequency was controlled by the motor's speed, as previously described (21). In brief, PDL fibroblasts were plated onto the deformable silicone dishes pre-coated with fibronectin (Sigma, St. Louis, MO, USA) (2 ng/ml in 0.5M NaCl-50mM Tris-HCl, pH 7.5), in normal glucose (5.5 mM) DMEM supplemented with 10% (v/v) FBS. Wherever indicated, 4 hours before mechanical deformation, the appropriate volume of D-glucose solution was added as to obtain a final concentration of 30 mM (final osmolality 330mOsm/Kg). Alternatively, a salt solution was added to reach an osmolality of 330mOsm/kg. Subsequently, cyclic strain [frequency 1 Hz and extension 8%, falling

within the range of physiological tissue deformation during orthodontic treatment (22)] was applied to the cells for the indicated time periods.

Western Immunoblot analysis

Protein expression was monitored by Western immunoblot analysis, as previously described (23). In brief, after stretching for the indicated time periods, protein extraction was performed in Laemmli sample buffer supplemented with protease- and phosphatase-inhibitor cocktails (Sigma). Protein samples were separated with SDS-PAGE, and the proteins were transferred to PVDF membranes (Amersham Biosciences, Buckinghamshire, UK). Western immunoblot analysis was performed with primary antibodies against p38, phospho-p38 (Thr180/Tyr182), phospho-JNK (Thr183/Tyr185), and JNK (Cell Signaling Technology, Hertfordshire, UK). The antibodies against panERK and phospho-ERK1/2 (Thr202/Tyr204) were from BD Pharmingen (Bedford, MA); the antibody against GAPDH was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Subsequently, the membranes were incubated with either goat anti-mouse or goat anti-rabbit secondary horseradish peroxidase-conjugated antibodies (Sigma) and the immunoreactive bands were visualized on Kodak-X-OMAT AR film by chemiluminescence (ECL kit), according to the manufacturer's instructions (GenScript, Piscataway, NJ, USA). In all cases, GAPDH expression was used as a loading control.

Estimation of intracellular levels of reactive oxygen species (ROS)

For the estimation of intracellular ROS levels in human PDLF under hyperglycemic conditions the 2',7'-dichlorofluorescein-diacetate (DCFH-DA) assay was employed, as previously described (24). In brief, cells were plated in 96 multi-well microplate in a normal glucose DMEM containing 10% (v/v) FBS. When cells reached confluency glucose was adapted to 30mM and 10μM DCFH-DA (Sigma, St. Louis, MO, USA) was added. 500μM H_2O_2 (Sigma) was added as a positive control. Fluorescence intensity (excitation wavelength: 485 nm, emission wavelength: 520 nm) was recorded using an Infinite 200 Tecan micro-titer plate photometer (Tecan Trading AG, Switzerland) at the indicated time intervals.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Gene expression analysis was performed by quantitative real-time polymerase chain reaction (qRT-PCR) as previously described (19). Total RNA was extracted using Trizol reagent (Life Technologies, Europe, BV). First-strand complementary DNA (cDNA) was synthesized using the PrimeScript^{TM} RT Reagent Kit according to the manufacturer's instructions (Takara Bio Inc, Tokyo, Japan) and subjected to qRT-PCR using the qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems Ltd, London, UK) in a MX3000P cycler (Stratagene, La Jolla, CA, USA). Data analysis was performed with MxPro QPCR software. Cycle threshold (C_t) values of each target gene were obtained and normalized to that of the house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The $\Delta\Delta C_t$ method was used to evaluate the relative mRNA expression levels of each gene. The primers used for amplification were *c-fos: forward 5′-AGA ATC CGA AGG GAA AGG AA-3′, reverse 5′-CTT CTC CTT CAG GTT GG-3′, ALP: forward 5′-GGC CAT TGG CAC CTG CCT TA-3′, reverse 5′-ACC CAT CCC ATC TCC CAG GAA-3′,OPN forward 5′-GCC GTG GGA AGG ACA GTT ATG-3′, reverse 5′-TGC TCA TTG CTC TCA TCA TTG G-3′, and GAPDH: forward 5′-GAG TCC ACT GGG GTC TTC-3′, reverse 5′-GCA TTG CTG ATG ATC TTG GG-3′.*

Mineralization assay

In order to determine hyperglycemic and hyperosmotic conditions' effect on PDL fibroblasts' ability towards osteoblastic differentiation Alizarin Red-S staining assay was employed (25). Briefly, human PDL fibroblasts were plated in a 12-well plate in DMEM supplemented with 10% (v/v) FBS. When cultures reached ~90% confluence medium was aspirated and replaced with the commercial osteo-inductive medium StemPro® Osteogenesis Differentiation kit (Gibco BRL, Invitrogen, Paisley, UK) for 21 days with medium changes every 3 days along with the appropriate volume of either D-Glucose solution so as to obtain a final concentration of 30 mM and a final osmolality of 330mOsm/kg, or with a salt solution (5M NaCl and 0.4M KCl) at the same final osmolality as that of hyperglycemic conditions, i.e. 330mOsm/kg. At the end of the incubation period with osteogenic medium, cells were fixed with 4% (v/v)

formaldehyde solution (Sigma) for 30 minutes and stained for 20 min with 40mM Alizarin Red-S pH 4.2, at room temperature with gentle rotation. Cultures were then rinsed with water, incubated for 15 min with PBS in order to reduce non-specific Alizarin Red-S stain and photographed under an inverted microscope. Quantification of calcium mineral content was estimated after extraction of Alizarin Red-S stain with 10% (w/v) cetylpyridinium chloride (CPC) (Sigma) in 10mM sodium phosphate, pH 7.0, for 15 min at room temperature. Alizarin Red-S concentration of extracts was determined by absorbance measurement at 562nm on an Infinite 200 Tecan micro-titer plate photometer (Tecan Trading AG, Switzerland).

Statistical analysis

All experiments were repeated at least three times. Values presented are the means ± standard deviations. Differences were considered statistically significant when $p < 0.05$ (Student's t test).

RESULTS

Exposure of human PDLF to high glucose attenuates the cyclic tensile stretching-mediated activation of *c-fos* expression.

Activation of *c-fos* gene is considered an immediate response of PDLF to mechanical loading (17, 26). Therefore, the effect of high glucose concentration on this particular response of human PDLF to cyclic tensile stretching was studied. Accordingly, PDLF were plated onto fibronectin-coated silicone dishes, in the presence of normal (5.5 mM) or high (30 mM) glucose concentrations and were subjected to uniaxial cyclic tensile stretching (extension 8% frequency 1 Hz) for 30 minutes. As can be seen in Figure 1A, *c-fos* is activated by cyclic tensile loading in fibroblasts that are cultured under normal glucose conditions. On the contrary, under high glucose conditions tensile stretching-induced *c-fos* gene activation is significantly attenuated.

Hyperglycemic conditions suppress cyclic tensile stretching-elicited up-regulation of osteoblast-specific genes.

Taking into consideration that periodontal ligament fibroblasts have an osteoblastic differentiation potential and given that mechanical forces applied to the tissue play a key role in the regulation of osteoblast-specific genes, the effect of hyperglycemic conditions on the cyclic tensile stretching-mediated up-regulation of osteoblast-specific genes' expression was examined. Accordingly, cyclic tensile stretching was applied to human PDLF, for an 18 hours-period either under normal or high glucose conditions. At the end of this period mRNA levels of alkaline phosphatase (*ALP)* and osteopontin (*OPN)*, two major markers of osteoblastic differentiation, were assessed by real-time qRT-PCR. It was found, that the application of cyclic tensile stretching resulted in a significant increase of *ALP* and *OPN* gene expression in fibroblasts incubated under normal glucose conditions. On the other hand, under hyperglycemic conditions the cyclic tensile stretching-induced increase of *ALP* and *OPN* mRNA levels was significantly inhibited (Fig.1B).

Exposure of periodontal ligament fibroblasts to hyperglycemic conditions do not provoke an increase in intracellular ROS levels.

Next, the mechanisms underlying the effect of high glucose concentrations on the reduced response of PDLF to mechanical stimulation were investigated. As several lines of evidence indicate that in diabetic patients hyperglycemic conditions may provoke an oxidative stress that may contribute to the increased bone loss and impaired function of cells capable for an osteoblastic differentiation (27-28), human PDLF were exposed to normal or high glucose concentration and the intracellular ROS levels were estimated by employing the DCFH-DA assay. As can be seen in Figure 2, incubation of cells up to 72 hours in culture medium supplemented with high glucose concentration did not increase intracellular ROS levels compared to periodontal ligament fibroblasts incubated under normal glucose conditions.

Hyperosmolality diminishes the cyclic tensile stretching-induced *c-fos* activation and weakens periodontal ligament fibroblasts' potential for an osteoblastic differentiation

Subsequently, it was assessed whether the increased osmolality caused by high glucose concentration could impact stretching-subjected periodontal ligament fibroblasts' immediate responses, such as *c-fos* activation. Indeed, although the normal glucose (5.5. mM) medium has an osmolality of 300mOsm/Kg, the presence of 30 mM of glucose increases the osmolality to 330mOsm/Kg. Therefore, PDLF were exposed to a control medium, to high glucose or to another hyperosmotic agent of different nature, i.e. a salt solution at the same final osmolality, i.e. 330mOsm/kg, and cells were subjected to cyclic tensile stretching. As can be seen in Figure 3A, although under control conditions mechanical stimulation leads to an increase of *c-fos* expression, in the presence of high glucose or high salt conditions *c-fos* expression was significantly attenuated. Exposure of cells to 30mM of the osmolyte sorbitol had the same inhibitory effect on *c-fos* expression (data not shown). Furthermore, the presence of either high glucose concentration or salt of the same final osmolality during an 18-hours' application of cyclic tensile loading reduced cyclic loading-elicited up-regulation of osteoblast-specific gene markers *ALP* and *OPN* (Fig.3B). In accordance, incubation of cells with an osteo-inductive medium along with high glucose concentration or salt of the same final osmolality, i.e. 330mOsm/kg, compromised PDL fibroblasts' capacity for an osteoblastic differentiation, as portrayed by their attenuated calcium mineral content assessed with Alizarin Red-S staining (Fig.4). The above indicate that it is most probably the increase of osmolality under hyperglycemic conditions that is responsible for the decreased response of human PDLF to mechanical stimulation.

Cyclic tensile stretching-mediated phosphorylation of ERK and JNK MAPK kinases is abolished under hyperosmotic conditions

It has been shown that mechanical stretching-induced *c-fos* overexpression in human PDLF is mediated by the upstream activation of the ERK and JNK, members of the MAPK family (29). Accordingly, the effect of hyperglycemic and hyperosmotic conditions on the activation of MAPK after mechanical stimulation was checked. To this end, prior to strain application, PDLF were exposed for 4 hours to a high glucose or a salt solution of the same final osmolality (330mOsm/kg). As shown in Figure 5, mechanical stimulation provokes an immediate (within 20 min) activation of all

MAPK, i.e. ERK, JNK and p38, as shown before (29). Interestingly, hyperglycemic conditions significantly reduce the phosphorylation of ERK and JNK, in accordance to the reduction in *c-fos* expression; on the other hand, p38 activation is not affected. In addition, similar results were obtained when cells were cultured in high salt conditions, suggesting that the effect of high glucose concentration on the reduced response of human PDLF to tensile stretching is most probably due to the increased osmolality.

Discussion

Diabetes mellitus is a chronic metabolic disorder manifested by abnormally high glucose levels in the blood. Consequently, diabetic patients are at high risk of developing serious complications such as impaired bone metabolism leading to a decline of bones' quality (1). In this context, there is a number of reports indicating the negative effects of high glucose on the ability of periodontal ligament's cells for an osteoblastic differentiation (27, 30-31). Furthermore, diabetes is implicated in the alteration of organization and properties of connective tissue, which in turn has an effect on the mechanical properties of the tissue, eventually affecting the response of ligaments to stress (18). PDL is exposed to multifaceted stress through masticatory forces which are primarily vertical but depending on the inclination of the tooth, not always perpendicular to the vertical axis of the crown, along with orthodontic forces which can be either tensile or compressive depending on the force application and the type of movement. In tensile loading pattern, collagen fibers undertake the loading thus shielding the matrix from being mechanically distorted (11). In this context, it has been indicated that the addition of collagenase in the rat PDL tissue, yielded a dose-response reduction in the mechanical properties of the PDL as indicated by reduced stiffness, smaller elongation capacity, lower strength and smaller toughness (32). This is important for orthodontic patients presenting with a systematic disease affecting the connective tissue homeostasis, especially diabetes, as their PDL is not capable of sustaining ordinary loads applied to the general population. However and to the best of our knowledge, there is no previous work investigating the immediate responses of the PDL fibroblasts to physiologically relevant mechanical strains- such as those applied to the periodontal tissue during therapeutic orthodontic treatment- under a hyperglycemic environment simulating the diabetic state.

Therefore, in the present study early passage human PDLF were exposed to a high glucose concentration, simulating the *in vivo* hyperglycemic diabetic state *in vitro*, and subsequently were subjected to cyclic mechanical stretching in order to study under hyperglycemic conditions the activation of *c-fos*, a central transcription factor regulating osteo-specific genes. In accordance with previous studies indicating that mechanical forces activate *c-fos* (17, 29, 33), under normal glucose conditions we found a notable up-regulation of *c-fos* expression, elicited by mechanical stimulation. On the contrary, incubation of cells in a hyperglycemic environment significantly attenuated the stretching-induced *c-fos* activation. In accordance, mechanical stimulation of PDLF under normal conditions leads to the up-regulation of two major osteogenic differentiation marker genes, i.e. *ALP* and *OPN*, which is annulled in the presence of high glucose conditions. Taken together, the above results indicate that exposure of PDLF to a high glucose concentration impairs their mechanical force-mediated immediate responses as well as their osteoblastic differentiation capacity, as observed by the reduction in *c-fos, ALP* and *OPN* expression, respectively, and consequently may compromise their ability for repair, remodeling and maintenance of periodontal tissue homeostasis. Our observations are in agreement with previous reports indicating decreased mineralized nodule formation in PDL cells grown under high glucose conditions (34-35). In addition, it has been shown that diabetic mice exhibited reduced bone formation in response to mechanical stimulation (36).

Since numerous studies have reported that oxidative stress is a major factor for the development of diabetic complications (4, 6), it was investigated whether high glucose concentration could provoke any changes in intracellular ROS levels in the cell model used in this study. To this end, we used a classical method for the evaluation of intracellular ROS, i.e. the DCFH-DA assay, which is based on the production of the highly fluorescent DCF after the oxidation by intracellular ROS (37). In contrast to previous publications (27-28, 31, 38), we did not observe any significant alterations in the intracellular ROS levels during incubation of the cells under hyperglycemic conditions from 15 minutes up to 72 hours. This discrepancy could be attributed to the different cell types used in the aforementioned reports compared to our study (human bone marrow-derived mesenchymal stem cells, mouse myoblast and mouse osteocyte-like cell lines versus human PDLF used in this study) (28, 38) or to the supraphysiological concentration of glucose (100mM) used (38) in contrast to the conditions simulating the diabetic state (30mM of glucose) used in our

study. In addition, the involvement of high glucose-induced hyperosmolality on the attenuation of cyclic stretching-mediated *c-fos* expression was investigated, since it has been reported that elevated glucose concentration contributes to diabetic complications through an osmotic stress (7). Interestingly, our experiments revealed that under high glucose or high salt conditions, mechanical stretching-elicited up-regulation of the osteogenic-related transcription factor *c-fos* is significantly diminished compared to normal glucose conditions. These results strongly indicate the implication of high glucose-mediated hyperosmolality in the impaired response of PDLF to mechanical stimulation.

Finally, the present study showed that in contrast to normal glucose state both high glucose and high salt conditions abolished the cyclic stretching-induced phosphorylation of ERK and JNK, in accordance with the attenuation of *c-fos* expression. On the other hand, p38 MAPK stretching-provoked phosphorylation was not affected by neither of the two osmotically active agents, i.e. glucose and salt. These findings suggest that PDLFs' inability to adequately respond to mechanical stimulation under hyperglycemic conditions arises from osmotic changes. Additionally, these results are in accordance with previous findings for the cyclic stretching-induced *c-fos* up-regulation in PDLF via the selective activation of ERK and JNK and not p38 MAPK (29). Interestingly, in another cell system, i.e. nucleus pulposus intervertebral disc cells, it has been shown that hyperosmolality, while activates p38 MAPK, inhibits the phosphorylation of ERK and JNK (39), in agreement with the data presented here. In addition, high glucose and hyperosmotic conditions diminish the mechanical stretching-mediated expression of *ALP* and *OPN*, as well as the osteoblastic differentiation of PDLF after a long-term incubation in an osteo-inductive medium.

In summary, the findings of the present study indicate that under high glucose conditions, such as those prevailing in the hyperglycemic diabetic state, human PDLF fail to adequately respond to mechanical stimuli, as can be seen by the inhibition of ERK and JNK phosphorylation and the attenuation of *c-fos, ALP* and *OPN* expression. *In vitro*, these data might have potentially important consequences for the homeostasis of PDL during tensile loading.

Conclusions

Impairment of immediate responses to mechanical loading under a hyperglycemic environment deteriorates PDLFs' ability for an osteoblastic differentiation, as exhibited by the reduction in osteoblastic differentiation gene markers' expression *ALP* and *OPN* and compromises their central role for the maintenance of periodontal tissue homeostasis, repair and remodeling. The above mentioned effects are most probably mediated by the increased osmolality observed in hyperglycemic conditions.

Conflict of interest

The authors declare no conflict of interest

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LEGENTS

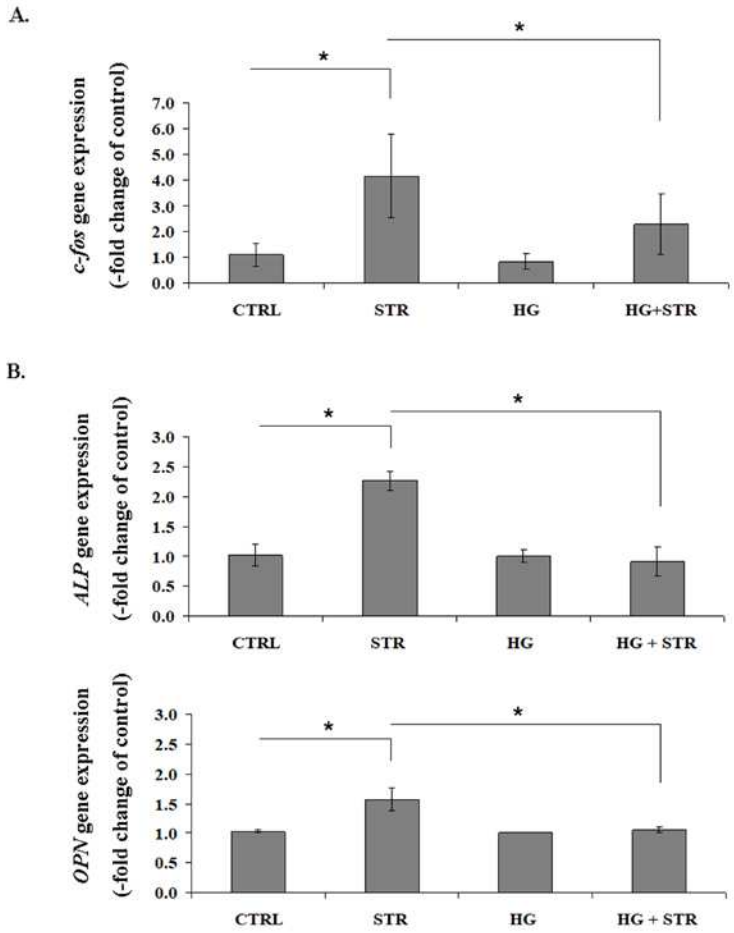

Figure 1. Incubation of human PDL fibroblasts under hyperglycemic conditions diminishes cyclic mechanical loading-induced activation of *c-fos* gene expression and osteogenic differentiation gene markers. Early passage human PDL fibroblasts, plated onto fibronectin-coated silicone dishes were incubated for 4 hours in a culture medium supplemented with 30mM D-glucose (HG) or in a culture medium supplemented with physiological, i.e. 5.5mM, glucose levels (CTRL). Subsequently, cells were subjected to cyclic tensile stretching (extension 8%, frequency 1Hz, STR) for 30 minutes (A), and for 18 hours (B). Total RNA was extracted and mRNA levels of *c-fos*, *ALP* and *OPN* were assessed by real-time qRT-PCR. Mean Ct values of all three genes were normalized to these of *GAPDH*. Relative gene expression was

calculated as the ratio of the expression level of treated cells to that of untreated control. qRT-PCR experiments were performed at least three independent times and from three different donors and results are expressed as mean values ± standard deviation. Asterisks denote statistically significant differences in comparison to the untreated control (Student's t-test, P <0.05).

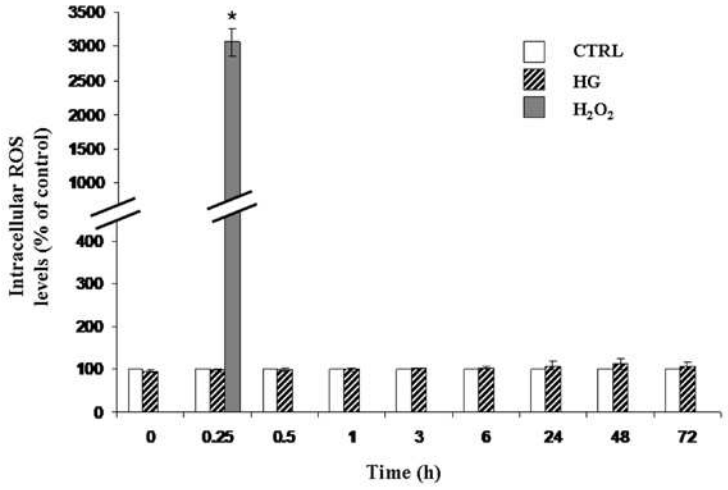

Figure 2. Evaluation of intracellular ROS levels of human PDL fibroblasts after short-term exposure to high glucose concentrations. Early passage human PDL fibroblasts were seeded in 96-well plate in normal glucose medium (CTRL) until confluency before the addition of high glucose at a final concentration of 30mM (HG) along with 10μM DCFH-DA. 500μM H_2O_2 was used as a positive control. Fluorescence intensity was recorded at 520nm at the indicated time intervals after the exposure of cells to high glucose, using a micro-titer plate photometer. Changes of ROS levels are expressed as the % ratio of untreated control. Data are expressed as the mean of two different experiments ± standard deviation.

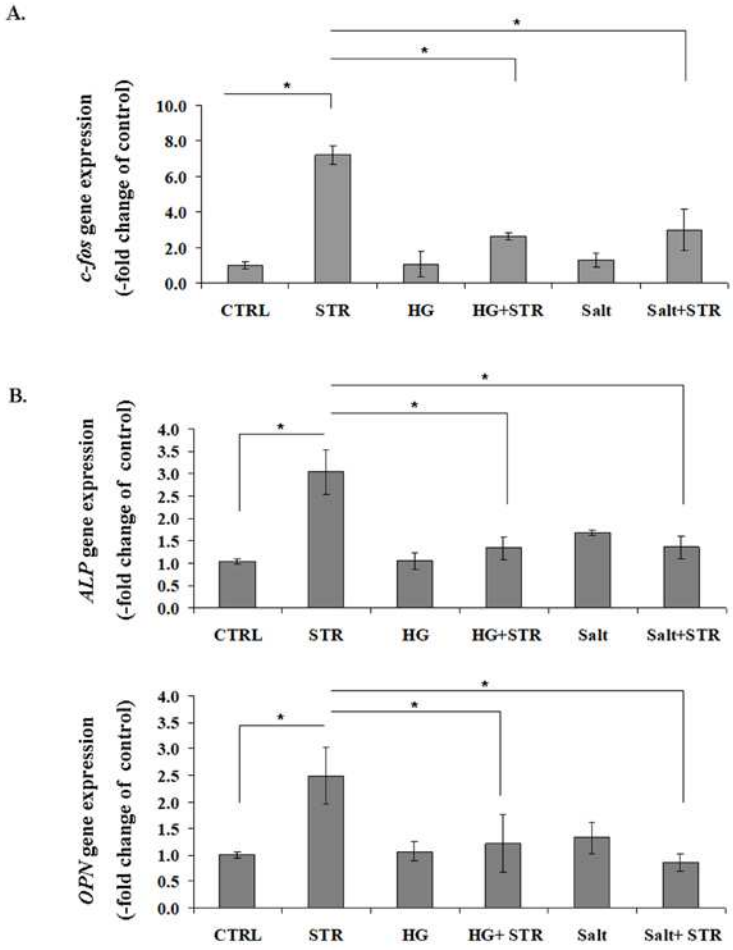

Figure 3. Cyclic tensile stretching-induced activation of *c-fos, ALP* and *OPN* gene expression of human PDL fibroblasts was attenuated by hyperosmotic stress caused by the increase of extracellular glucose concentration. Early passage human PDL fibroblasts, plated onto fibronectin-coated silicone dishes, were incubated for 4 hours under hyperosmotic conditions with a final osmolality of 330 mOsm/kg caused either by 30mM D-glucose (HG) or by a salt solution (5M NaCl and 0.4M KCl, Salt) at the same osmolality. Subsequently, cells were subjected to cyclic tensile stretching (extension 8%, frequency 1Hz, STR) for 30min (A), and 18 hours (B). Total RNA

was extracted and *c-fos*, *ALP* and *OPN* gene expression was evaluated by real-time qRT-PCR. Mean Ct values of all three genes were normalized to these of *GAPDH*. Relative gene expression was calculated as the ratio of the expression level of treated cells to that of untreated control. qRT-PCR experiments were performed at least three independent times and results are expressed as mean values ± standard deviation. Asterisks denote statistically significant differences in comparison to the untreated control (Student's t-test, $P < 0.05$).

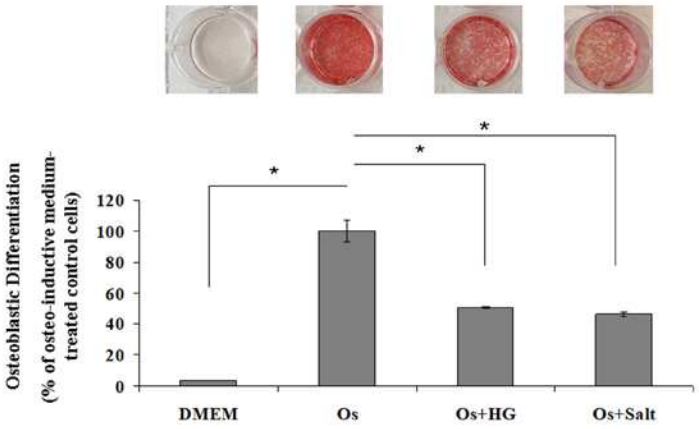

Figure 4. Hyperglycemic and hyperosmotic conditions diminish human PDL fibroblasts' capacity towards osteoblastic differentiation. Human PDL fibroblasts were incubated for 21 days either with an osteo-inductive medium alone (Os) or with the osteo-inductive medium along with high glucose (30mM-330mOsm/kg, Os+HG) or with a salt solution (5M NaCl and 0.4M KCl) at the same final osmolality (330mOsm/kg, Os+Salt). PDL fibroblasts cultured for the same time period with DMEM supplemented with 10% (v/v) FBS were used as the negative controls (DMEM). At the end of the 21-days' incubation period cultures were stained with Alizarin Red-S and calcium mineral content was assessed following extraction of Alizarin Red-S with 10% (w/v) cetylpyridinium chloride and absorbance measurement at 562nm with micro-titer plate photometer. Osteoblastic differentiation is expressed as a % ratio of the cells cultured with the osteo-inductive medium alone.

Results are expressed as means values ± standard deviation. Asterisk denotes statistically significant difference in comparison to the osteo-inductive medium-treated control cells (Os).

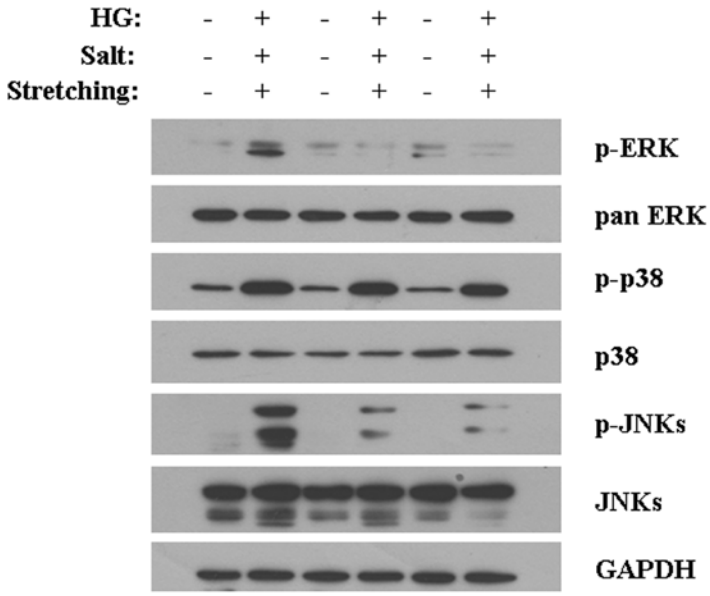

Figure 5. Elevation of extracellular glucose concentration prevents cyclic tensile stretching-elicited activation of ERK and JNK MAPK kinases. Early passage human PDL fibroblasts, plated onto fibronectin-coated silicone dishes, were treated for 4 hours with 30mM D-glucose (HG, 330mOsm/kg) or a salt solution (5M NaCl and 0.4M KCl, Salt) both having an osmolality of 330mOsm/kg. Cells incubated under normal glucose conditions, i.e. 5.5mM, served as a control. Subsequently, cells were subjected to cyclic tensile stretching (extension 8%, frequency 1Hz) for 20min (STR), cell lysates were collected and the activation of ERK, JNK and p38 MAPK kinases was assessed by Western blot analysis. GAPDH was used as a loading control. Representative blots from 3 similar experiments are depicted.